Free Radical Biology and Medicine 117 (2018) 37–44

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Silencing of long isoforms of nuclear factor erythroid 2 like 1 primes macrophages towards M1 polarization

Huihui Wang[a], Jiayu Zhu[a], Zhiyuan Liu[a], Hang Lv[a], Peng Lv[b,c], Feng Chen[d], Jingqi Fu[a], Yongyong Hou[a], Rui Zhao[a], Yuanyuan Xu[a], Qiang Zhang[e], Jingbo Pi[a,b,*]

[a] Program of Environmental Toxicology, School of Public Health, China Medical University, No. 77 Puhe Road, Shenyang North New Area, Shenyang 110122, China
[b] The Hamner Institutes for Health Sciences, 6 Davis Drive, Research Triangle Park, NC, 27709, USA
[c] Chinese Medical Association, 42 Dongsi Xidajie, Beijing 100710,China
[d] Department of Interventional Radiology, The First Affiliated Hospital of China Medical University, No. 155 Nanjing North Road, Heping District, Shenyang 110001, China
[e] Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, Georgia 30322, USA

ARTICLE INFO

Keywords:
Nfe2l1
Macrophage
Inflammatory response
Cytokine

ABSTRACT

Macrophages are a major component of the immune system and play an important role in regulating the magnitude, duration, and quality of the inflammatory response. Dissecting the functions of transcription factors regulating macrophage activation is important for understanding the inflammatory responses. Nuclear factor erythroid 2 like 1 (NFE2L1, also known as Nrf1) is a CNC-bZIP protein, which has multiple isoforms. While the exact physiological functions of various isoforms of NFE2L1 are still under investigation, accumulating evidence indicate that long isoforms of NFE2L1 (NFE2L1(L)) are important regulators in the antioxidant response, proteasome homeostasis and inflammation. In this study, we found that NFE2L1(L) was upregulated in response to LPS stimulation in RAW264.7 macrophages. Stable knockdown of *Nfe2l1*(L) (*Nfe2l1*(L)-KD) in RAW264.7 cells resulted in increased expression of multiple genes indicative of M1 polarization, including *Il6, Il1β, Cox2*, and *Ccl2*, under both resting and LPS-challenged conditions. In addition, lentiviral shRNA-mediated silencing of *NFE2L1*(L) in human monocytic SC and THP1 cells also significantly increased mRNA expression of *IL6, IL1β,* and *TNFα*. Furthermore, transient silence of *NFE2L1*(L) in primary human monocytes isolated from peripheral blood by nucleofection with small interfering RNA resulted in increased expression of *IL6* and *TNFα*. Analysis of the key transcription factors involved in M1 polarization revealed that *Nfe2l1*(L)-KD RAW264.7 cells have increased mRNA and protein expression and phosphorylation of STAT1 and STAT3 under both resting and M1 polarized conditions. Activation of the NFκB, ERK1/2 and p38 pathways in response to LPS was not affected by the reduction of NFE2L1(L). Moreover, *Nfe2l1*(L)-KD cells were found to have elevated levels of intracellular ROS, but macrophage M1 polarization induced by *Nfe2l1*(L) silence was independent of ROS accumulation. Collectively, our results show that knockdown of *Nfe2l1*(L) leads macrophages to M1 polarization by disinhibition of STAT1/3, and not through the NFκB, ERK1/2 and/or p38 signaling pathways. These findings indicate that NFE2L1(L) functions as a negative regulator of M1 polarization and pro-inflammatory response in macrophages.

1. Introduction

Macrophages are a main component of the immune system and play an important role in regulating the magnitude, duration, and quality of inflammatory response [1]. Two distinct functional phenotypes of macrophages have been categorized and described [2–4]. Classically-activated macrophages (M1 macrophages) play roles in producing pro-inflammatory cytokines, killing intracellular pathogens, and initiating

Abbreviations: ARE, antioxidant response element; ARG1, arginase 1; CCL, chemokine (C-C motif) ligand; CNC-bZIP, Cap 'n' Collar basic region leucine zipper; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; MRC1, macrophage mannose receptor 1; NFE2L1, Nuclear factor erythroid 2 like 1; NFE2L1(L), Long isoforms of NFE2L1; NOS2, nitric oxide synthase 2, inducible; PBS, phosphate-buffered saline; RETNLα, resistin-like-α; ROS, reactive oxygen species; RT-qPCR, reverse transcription quantitative real-time PCR; STAT, signal transducer and activator of transcription; TLRs, Toll-like receptors; TNFα, tumor necrosis factor α

* Corresponding author at: Program of Environmental Toxicology, School of Public Health, China Medical University, No. 77 Puhe Road, Shenyang North New Area, Shenyang 110122, China.
E-mail addresses: jbpi@cmu.edu.cn, jingbopi@163.com (J. Pi).

https://doi.org/10.1016/j.freeradbiomed.2018.01.022
Received 16 June 2017; Received in revised form 6 January 2018; Accepted 20 January 2018

the adaptive immune response. M1 macrophages produce high levels of interleukin (IL) 6, IL1β, nitric oxide synthase 2 (NOS2), tumor necrosis factor α (TNFα) and chemokine [C-C motif] ligand 2 (CCL2), and release high bactericidal levels of reactive oxygen species (ROS). Alternatively-activated macrophages (M2 macrophages) play roles in producing anti-inflammatory cytokines, tissue remodeling, and tissue repair. M2 macrophages have upregulated expression of arginase 1 (ARG1), macrophage mannose receptor 1 (MRC1) and resistin-like-α (RETNLα; also known as FIZZ1) [5,6]. These two cellular fates are determined by the response to the activation of Toll-like receptors (TLRs) and cytokines/interferon (IFN) γ, and to IL4 and IL13, respectively. Thus, macrophages are involved in both the destruction and regeneration of the inflamed tissues [7,8].

Nuclear factor erythroid 2 like 1 (NFE2L1, also known as NRF1) belongs to the Cap 'n' Collar basic region leucine zipper (CNC-bZIP) transcription factor family, and is ubiquitously expressed across tissues and cell types. NFE2L1 regulates a range of cellular functions including the antioxidant response, differentiation, metabolism, proteasome homeostasis and inflammatory response [9–18]. Human and rodent *Nfe2l1* genes can be transcribed into alternatively-spliced forms, resulting in multiple protein isoforms. Even though their consensus binding sequences are similar, each of the NFE2L1 isoforms may act differently and regulate a different set of genes, even exhibiting combinational or competitive effects [9,14,19–21]. The exact physiological functions of the various isoforms of NFE2L1 are still under investigation, but accumulating evidence indicates that the long isoforms of NFE2L1 (NFE2L1(L), also referred as TCF11) are an important regulator of the antioxidant response and proteasome homeostasis [9,22]. Far fewer studies are published on the role of the various isoforms of NFE2L1 in inflammatory responses [23–25]. NOS2, a transcriptionally-regulated enzyme that synthesizes nitric oxide (NO), participates in inflammatory responses by producing NO as an innate immune defense mechanism. TCF11 bound to small MafG protein is a negative regulator of the *NOS2* gene in human smooth muscle cells. Furthermore, transforming growth factor-β (TGFβ) treatment of these cells resulted in decreased expression of NOS2, an event that is dependent on TCF11/MafG binding to the promoter region of *NOS2* [25]. NFE2L1 splice variants were reported to participate in the induction of TNFα in murine dendritic cells and mast cells [23,24]. However, the expression and function of various NFE2L1 isoforms in macrophages have not yet been reported.

To investigate the function of NFE2L1(L) in inflammation, we developed stable knockdown of *Nfe2l1*(L) (*Nfe2l1*(L)-KD) in RAW264.7 and THP-1 cell cultures. We found that knockdown of *Nfe2l1*(L) resulted in increased expression of M1-associated pro-inflammatory cytokines and chemokines under basal and lipopolysaccharide (LPS)-challenged conditions, and is associated with enhanced STAT1/3 gene expression and activation. These findings identify NFE2L1(L) as a negative regulator of macrophage activation and M1 polarization.

2. Materials and methods

2.1. Cell culture and reagents

Mouse leukemic monocyte/macrophage cells (RAW264.7, ATCC TIB 71), human monocytic leukemia cells (THP-1, ATCC TIB 202) and immortalized human monocyte/macrophage cells (SC, ATCC CRL-9855) were purchased from American Type Culture Collection (ATCC, Manassas, VA). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS), 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. THP-1 cells were grown in the RPMI 1640 medium (ATCC) with 0.05% β-mercaptoethanol, 10% HIFBS, 100 U/ml penicillin and 100 μg/ml streptomycin. SC cells were cultured in Iscove's modified Dulbecco's medium (IMDM, ATCC30-2005) supplemented with 50 μM β-mercaptoethanol, 100 μM hypoxanthine, 16 μM thymidine and 10% HIFBS. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Phosphate buffered saline (PBS, pH 7.4) and supplements for cell culture were purchased from Life Technologies (Grand Island, NY). All other reagents including LPS (L6529), puromycin (P8833) and N-Acetyl-L-cysteine (NAC, A9165) were obtained from Sigma-Aldrich (St. Louis, MO). IL4 and IL13 were purchased from R&D systems (Minneapolis, MN). Fluorescent probe CM-H2DCFDA was purchased from Molecular Probes (Eugene, OR).

2.2. Human blood monocytes isolation

Peripheral blood samples were collected from healthy volunteers after signing the consent form. Fifty milliliters of peripheral blood was collected into EDTA coated vacuum blood collection tube. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. Human blood monocytes were purified from PBMC by negative selection using Dynabeads® Untouched™ Human Monocytes kit (Invitrogen, Life Technologies). Monocytes were cultured in IMDM (12–722F, Lonza, Germany) supplemented with 10% HIFBS. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. siRNA delivery by Nucleofection

Nucleofection was performed using the Amaxa® Human Monocyte Nucleofector® Kit (VPA-1007, Lonza) according to the manufacturer's protocol. In brief, blood monocytes or SC cells (1–2 × 10^6 cells) were resuspended in 100 μl of Human Monocyte Nucleofector Solution with 30 pmol of siRNA and transferred into a nucleofection cuvette. Electroporation was performed in the Nucleofector 2b device (Lonza) using the Y-001 program. After transfection, 500 μl of pre-equilibrated culture medium was added into the cuvette. Transfected cells were transferred into 12 well-plate containing pre-warmed transfection medium. After 24 h culture, cells were harvest for RNA extraction. The following small interfering RNA (siRNA) were used: Silencer™ Select Pre-Designed siRNA target human NFE2L1 (s9490) and Silencer™ Select Negative Control No. 1 siRNA.

2.4. Lentiviral-based short-hairpin RNA (shRNA) transduction

We obtained MISSION shRNA lentiviral vectors from Sigma-Aldrich. Transduction of RAW264.7 cells with lentiviral particles of shRNAs targeting mouse *Nfe2l1*(L) (SHCLND-NM_008686) or Scrambled non-target negative control (sh-Scr; SHC002V) was performed as described previously [26]. Transduction of THP-1 cells and SC cells with lentivirus-based shRNAs targeting human *NFE2L1*(L) (SHCLND-NM_003204) or sh-Scr was performed as described previously [22]. The selective media for RAW264.7 cells and THP-1 cells contained puromycin at 5.0 μg/ml and 0.6 μg/ml, respectively. Stable cell lines were continuously grown in the media containing the same concentrations of puromycin.

2.5. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was isolated with TRIzol® Reagent (Invitrogen, Life Technologies). RT-qPCR was performed as described previously [27]. Total RNA was reversely transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A GoTaq qPCR Master Mix reagent system (Promega, Madison, WI) for qPCR was used. Real-time fluorescence detection achieved using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Primers (sequences are shown in Table S1) were designed by using Primer Express 4 (Life Technologies, Shanghai, China) and synthesized by Life Technologies.

2.6. Western blot analysis

Collection of cell lysates and Western blotting were performed as